PLNT2530 Plant Biotechnology
2024
Unit 8b

Direct Transformation with DNA

Transformation by direct transfection of DNA into plants

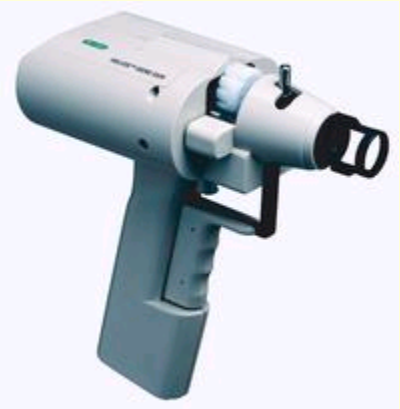
- It has long been observed that cells have mechanisms for incorporating free DNA into their chromosomes.

- This seems to be an effect of the naturally-occurring DNA repair mechanisms.

- Foreign DNA will often be integrated at random chromosomal sites

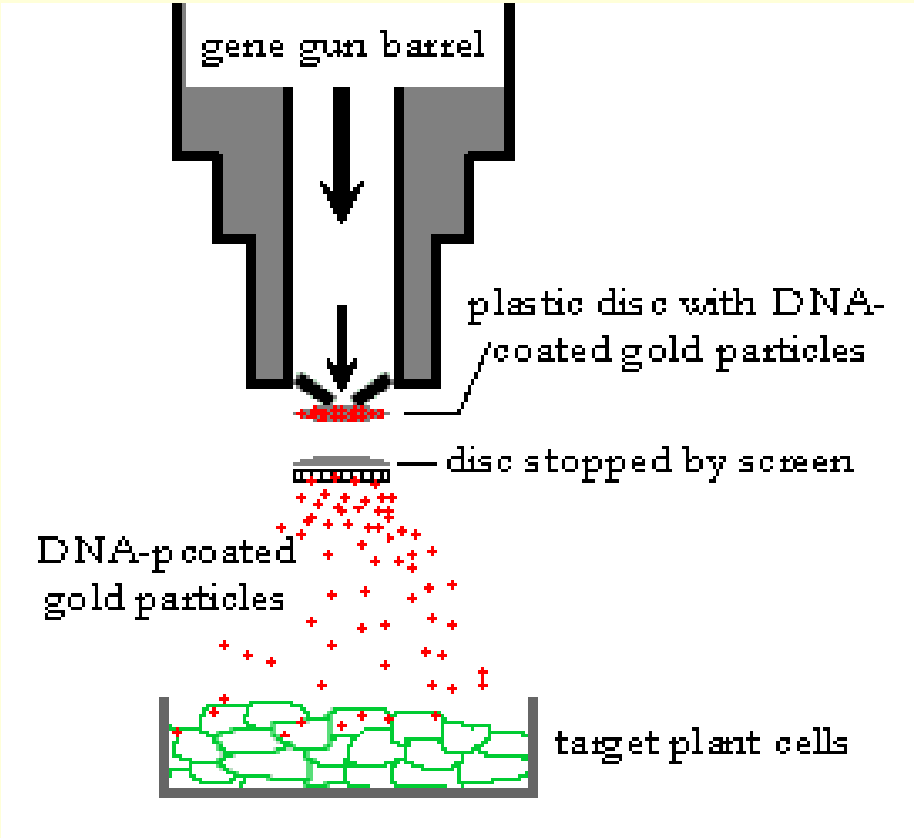
- Therefore, if you can find a way to get DNA into the cell, you can probably transform the cell.

Gene Gun



- Known as The "Gene Gun" method.
- Also referred to as micro-projectile bombardment or biolistics (ballistics using biological components).
- This technique is used for in vivo transformation and has been especially useful in transforming monocot species like corn and rice.

A Gene Gun shoots genes into plant cells. DNA is coated onto small particles of gold or tungsten approximately two microns in diameter. The particles are placed in a vacuum chamber and the plant tissue is placed below the chamber. The particles are propelled at high velocity using a short pulse of high pressure Helium gas into any target cell or tissue.



Two types of Gene Guns

Fixed gun for cell culture

Handheld gun for in-planta transformation

The real trick is to fire pellets with enough force to break into cell, but not so much as to go right through tissue. Must optimize distance, placement of tissue, dispersal of charge (don't want charge to all go in one place).

Advantages of "Gene Gun"

- no host range problems
- can transform intact tissue, eliminating de-differentiation, somaclonal variation problems associated with callose or protoplasts

Disadvantages of "Gene Gun"

- Has to be optimized for each tissue type
- Can only transform superficial cells
- very hard to target the specific cell layer you want

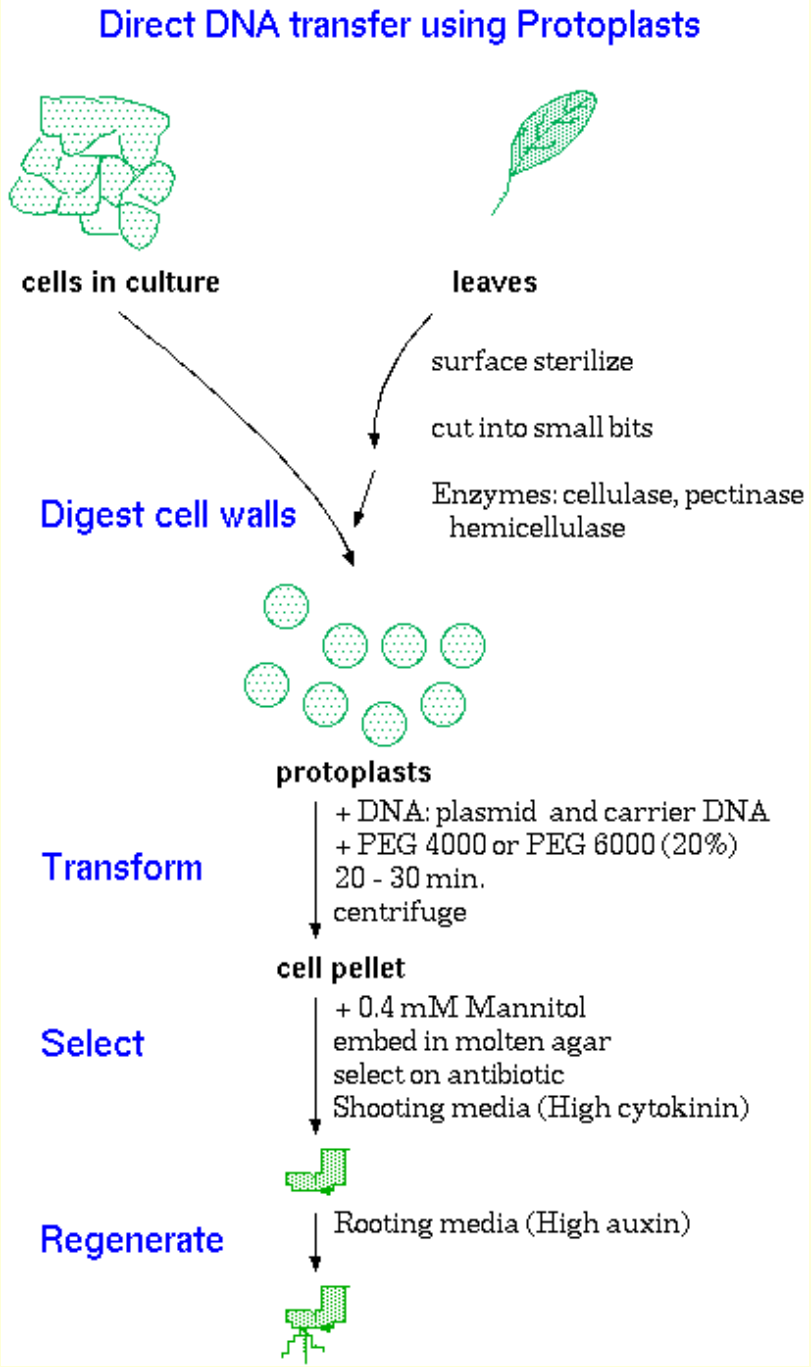
Protoplast Transformation

Advantages

- Not limited to species infected by *A. tumefaciens*
- No need to clone constructs into T-DNA based vector; No cocultivation with *A. t.*

Disadvantages

- Protoplasts very delicate; difficult to obtain



Antibiotic resistance assay

It is often possible to screen plantlets for presence or absence of a transgene using the kanamycin resistance gene for selection. In transgenic *B. napus,* transformed plants expressing KanR show full root growth when plantlets are geminated and grown on media containing Kanamycin (left). Root growth of non-transformed plants is almost completely inhibited (right).

[Peijun Zhang, Ph.D. thesis, University of Manitoba, 1998].

Transgenic Kan^R plants

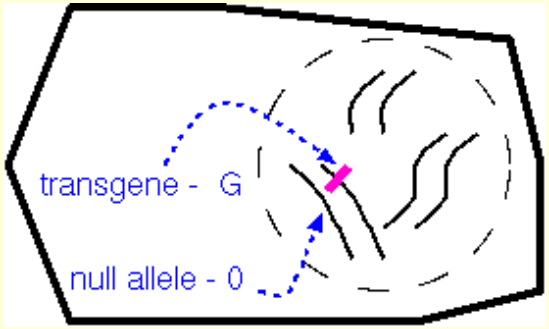
Untransformed control plants

Selection of homozygous transformed lines

For many practical purposes, lines **single copy**, **homozygous** for the transgene are needed.

- With multiple copy numbers, gene silencing often occurs. Gene silencing often results from high expression levels of an mRNA transcript.
- When transgenic loci are not homozygous, progeny segregate for the transgene. Progeny in the initial transformant generation, T_0, are hemizygous* Result: <u>some segregating progeny in later generations will not have the trait!</u>

***hemizygous** - only one of the two copies of a chromosome will get the transgene (G). The other copy, referred to as the null allele (0), will not.



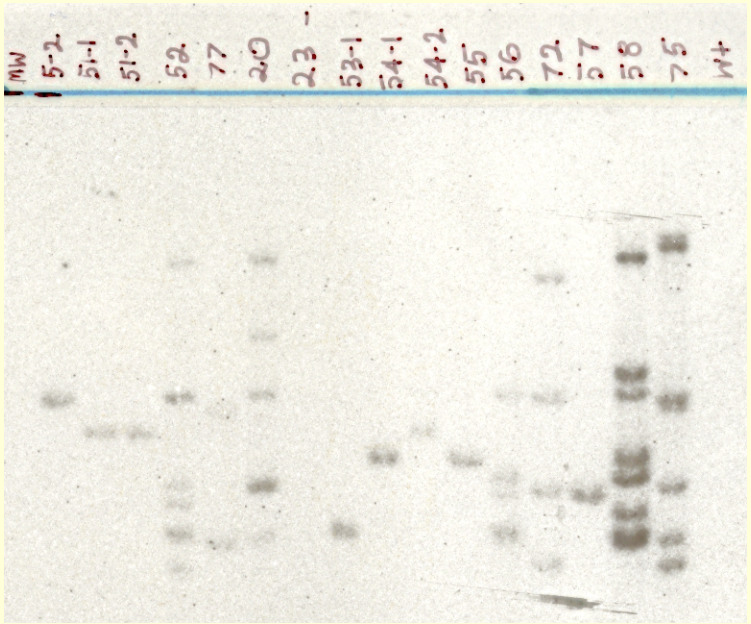
Determining transgene copy number by Southern blot

Most genes are smaller than the average fragment size for a 6-cutter restriction endonuclease (~ 4096 bp)

Therefore, if there is only one copy of a gene, we expect to see a single band on a Southern blot.

For each transgenic locus, there is usually a single band

Therefore, the number of bands is a good estimate of the transgene copy number.



Southern blot of T1 transgenic Brassica napus transformed for pea PR10.
Dept. of Plant Science, Univ. of Manitoba

Identification of homozygous transformed lines

The conventional way to determine homozygosity is through Mendelian genetics.

- The progeny of parents from T1 or later generations can be screened for segregation of the transgene.
- If the plant has a single-copy insertion, homozygous parental lines can be identified by lack of segregation for the transgene.
- It is usually safest to assay directly for the gene of interest (eg. by PCR) rather than relying on the selective marker (eg. KanR). This is because one can often get partial T-DNA insertions, missing the gene of interest.

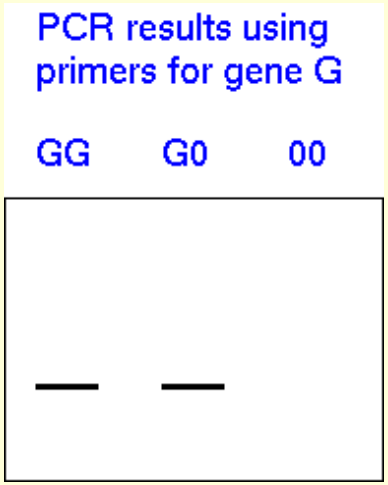
If we self T_0

G0 x G0

↓

T_1 GG : G0 : 00 (1 : 2 : 1) — Genotype

G : 0 (3 : 1) — Phenotype



It's easy to screen for G_ plants using PCR, and looking for presence of the gene. But how do we find out <u>which plants are GG and which are G0</u>?

To identify individual plants that are homozygous for the transgene:

1) Grow **10 - 20 single copy plants** until several true leaves are available, but well before flowering.
2) **Extract DNA** from a leaf taken from each individual,
3) Test for presence of the transgene by **PCR**. Use primers specific for the transgene, not the vector.
4) **Discard null segregants**, which do not give a band, and bag positive plants at flowering to prevent cross-pollination.
5) **Collect seed from each parent** and keep in separate envelopes.
6) For 5 positive parents, **grow at least 15* plants** until several true leaves are available.
7) Extract DNA from each individual, and test for the presence of the transgene by **PCR**. (You may save some work by freezing leaf tissue for 5 sets of progeny, but only testing one individual at a time, until you get a true homozygote).

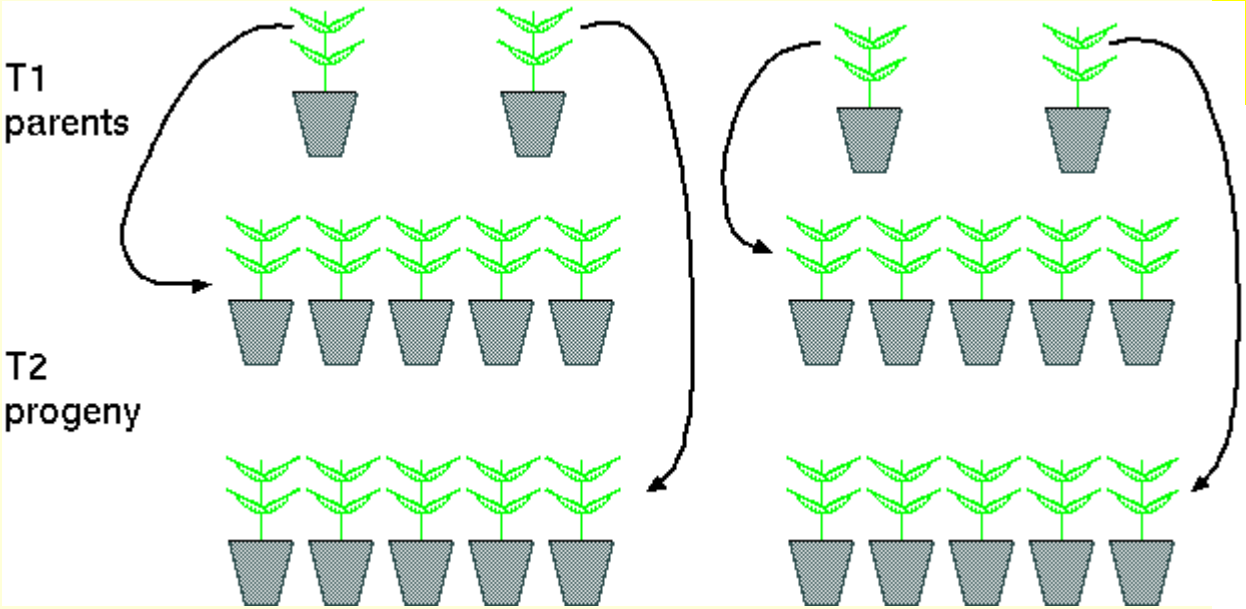
If any line tests positive in all individuals, that line must be homozygous. If some individuals test negative, it may be because of bad DNA preps. It is sometimes worth re-doing the DNA preps and doing PCR on the new DNA.

*How many progeny need to be tested, for each transgenic line, to choose a homozygous parent?

We want to eliminate the possibility that a parent testing positive for G is heterozygous

- 2/3 of all positive parents will turn out to be heterozygous
- For any heterozygous parent, the probability that any single offspring will test positive is 0.75.

P(heterozygous parent | all N T2 progeny test positive) = 2/3 (0.75^N)



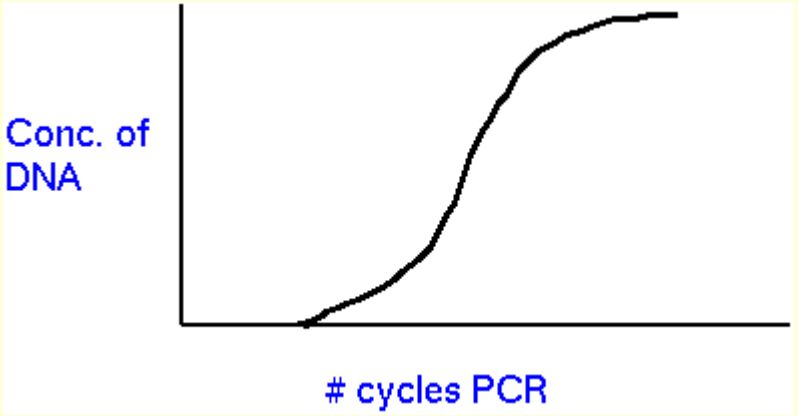
N	P
1	0.500
5	0.158
10	0.038
15	0.009
20	0.002

Identification of homozygous lines by qPCR

Jos C Mieog*, Crispin A Howitt and Jean-Philippe Ral (2013) Fast-tracking development of homozygous transgenic cereal lines using a simple and highly flexible real-time PCR assay. BMC Plant Biology 2013, 13:71 doi:10.1186/1471-2229-13-71

http://www.biomedcentral.com/1471-2229/13/71

qPCR (quantitative PCR) - a method for quatifying DNA or RNA by carefully-controlled PCR reactions.



Amplification of DNA by PCR is not linear with respect to cycles. Very little DNA is produced in early cycles, but increase is exponential over time. As reaction components are exhausted, the amplification curve reaches a shoulder, with little additional amplification.

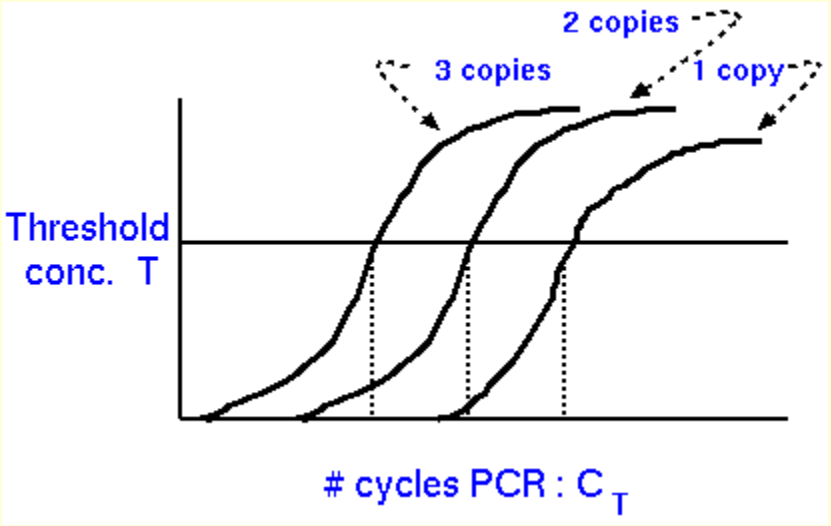
Quantitative PCR is routinely used to quantify input DNA. Quantitation reqires:

- control DNAs whose concentration is known precisely
- replicate amplifications to measure the standard error of the PCR reaction

Identification of homozygous lines by qPCR

The more copies of a sequence, the fewer cycles of PCR are required to reach an arbitrary threshold concentration of PCR product, C_T.

We can quantify DNA relative to controls by comparing the number of cycles at which controls reach C_T, with the number of cycles at which unknowns reach C_T.



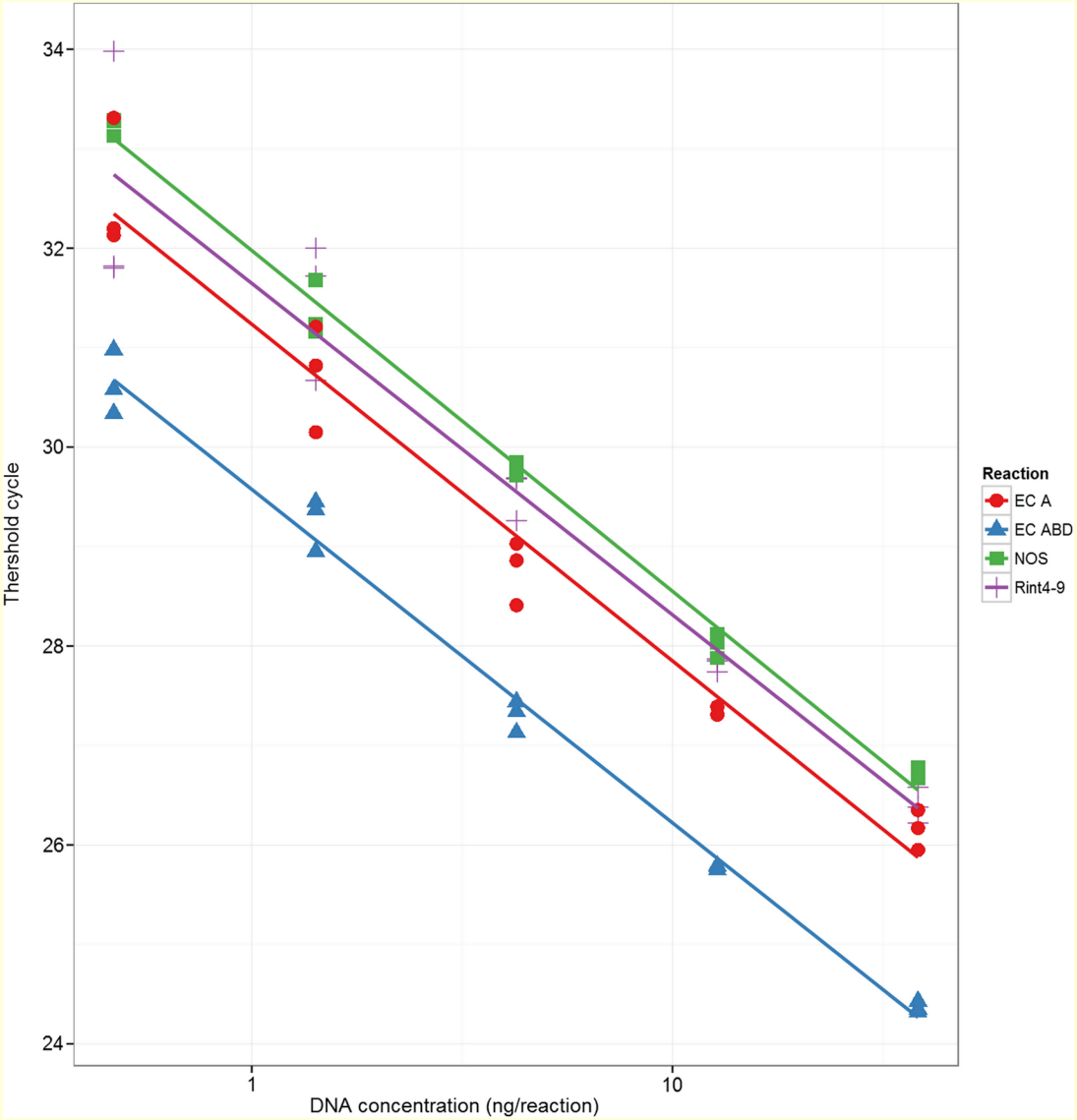
The copy number for a gene of interest can then be calculated by the formula:

$$\text{copy number} = 2^{[C_T\ (\text{reference}) - C_T(\text{Gene-of-Interest})]} \times (\text{number of reference gene copies})$$

Identification of homozygous lines by qPCR

To accurately measure C_T , samples of transgenic wheat were amplified at 5 different concentrations, three replicates each.

<u>primer pairs amplify:</u>
NOS -transgene
ECA - EC* from A genome only (single copy control)
ECABD - EC from A,B and D genomes (3-copy control)
Rint9 - rice intron, found in transgene



EC* - wheat Epsilon Cyclase, present in A,B and D genomes

Identification of homozygous lines by qPCR

T_0 plant has 1 copy: look for homozygous segregants in T_1

T_0 plant has 3 copies: look for homozygous segregants in T_2

T_0 plant has 7 copies: look for homozygous segregants in T_2